TITLE:

The importance of connexin hemichannels during chondroprogenitor cell differentiation in hydrogel versus microtissue culture models.

BRIEF RUNNING TITLE: … Hemichannels in chondroprogenitor differentiation

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ABBREVIATIONS:

BM - MSCs: bone marrow mesenchymal stromal cells
18αGCA/GCA: 18-α glycyrrhetinic acid
ATP: adenosine-5'-triphosphate
GAG: glycosaminoglycan
3D: three dimensional

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Gap junction

Abstract

Appropriate selection of scaffold architecture is a key challenge in cartilage tissue engineering. Gap junction-mediated intercellular contacts play important roles in pre-cartilage condensation of mesenchymal cells. However, scaffold architecture could potentially restrict cell-cell communication and differentiation. This is particularly important when choosing the appropriate culture platform as well as scaffold-based strategy for clinical translation, *i.e.*, hydrogel or microtissues, for investigating differentiation of chondroprogenitor cells in cartilage tissue engineering. We, therefore, studied the influence of gap junction-mediated cell-cell communication on chondrogenesis of bone marrow-derived mesenchymal stromal cells (BM - MSCs) and articular chondrocytes. Expanded human chondrocytes and BM - MSCs were (re-)differentiated either in micromass cell pellets or encapsulated as isolated cells in alginate hydrogels. Samples were treated with and without the gap junction inhibitor 18-α glycyrrhetinic acid (18αGCA). DNA and glycosaminoglycan (GAG) content and gene expression levels (collagen I/II/X, aggrecan, connexin 43) were quantified at various time points. Protein localization was determined using immunofluorescence, and adenosine-5'-triphosphate (ATP) was measured in conditioned media. While GAG/DNA was higher in alginate compared to pellets for chondrocytes, there were no differences in chondrogenic gene expression between culture models. Gap junction blocking reduced collagen II and extracellular ATP in all chondrocyte cultures and in BM - MSC hydrogels. However, differentiation capacity was not abolished completely by 18αGCA. Connexin 43 levels were high throughout chondrocyte cultures and peaked only later during BM - MSC differentiation consistent with the delayed response of BM - MSCs to 18αGCA. Alginate hydrogels and microtissues are equally suited culture platforms for the chondrogenic (re-)differentiation of expanded human articular chondrocytes and BM - MSCs. Therefore, reducing direct cell-cell contacts does not affect *in vitro* chondrogenesis. However, blocking gap junctions compromises cell differentiation, pointing to a prominent role for hemichannel function in this process. Therefore, scaffold design strategies which promote an increasing distance between single chondroprogenitor cells do not restrict their differentiation potential in tissue-engineered constructs.

Introduction

Damage to articular cartilage due to injuries or diseases frequently leads to a loss of the tissue's structure and function. This causes pain and impaired mobility for affected people and puts a significant financial burden on health care systems and economies worldwide. Despite major advances in cartilage tissue engineering aimed at growing the patient's own cells on scaffolding materials for subsequent re-transplantation, the reliable, functional repair or regeneration of articular cartilage remains a challenge (1). Key challenges in cartilage tissue engineering include the appropriate selection of cell source and scaffold architecture.

Multipotent mesenchymal stromal cells from human bone marrow (BM - MSC) and chondrocytes isolated from articular cartilage biopsies are currently the main sources for chondroprogenitor cells. Yet, we are still lacking appropriate culture protocols to initiate stable chondrogenic phenotypes of these cell types *in vitro*. For example, articular chondrocytes, in particular, when harvested from clinically relevant elderly patients, rapidly dedifferentiate during *ex vivo* propagation and lose their chondrogenic phenotype (2). Unfortunately, this process seems only partially reversible under *in vitro* conditions as seen by sustained high levels of collagen type I, a marker of inferior fibrocartilage formation (2, 3). BM - MSCs, on the other hand, tend to differentiate in a process more reminiscent of endochondral ossification including the onset of tissue hypertrophy and calcification (4). In order to overcome some of these issues and to support the *ex vivo* differentiation of chondroprogenitor cells and the formation of functional cartilage tissue, a wide range of approaches for cell culture have been developed that could broadly be classified into scaffold-/matrix-based and biomaterial-free techniques (5, 6). Among the former, hydrogels are very popular matrices for cartilage tissue engineering as they provide a three dimensional (3D) culture environment favorable for chondrogenic differentiation (7-9). The simplest and most widely used scaffold-free method is micromass pellet or microtissue culture, in which cell pellets are formed by centrifugation allowing for extensive cell-cell communication at the start of the culture (10, 11). This may be important, in particular, in the early stages of cell differentiation, considering the importance of cell-cell contacts to initiate chondrogenesis during pre-cartilage condensation of mesenchymal cells in skeletogenesis (12). However, the architecture of hydrogels, where the majority of cells are spatially separated by the hydrogel matrix, seems to inherently restrict direct cell-cell contacts and could potentially affect chondrogenic differentiation.

Intercellular communication during early chondrogenesis occurs via cell adhesion molecules, such as N-cadherin and Neural Cell Adhesion Molecule (N-CAM), or gap junctions (13, 14). While the first two molecules disappear in the course of chondrogenesis (13), gap junctions can still be found in mature cartilage (15). Gap junctions are intercellular channels formed by connexins that allow for rapid exchange of ions, metabolites and other small molecule messengers (16). They are expressed in virtually all tissues and are involved in various cellular functions including cell growth, differentiation and death. One half of a gap junction channel that is not connected to an adjacent cell can still be functional and is termed a hemichannel. The major member of the connexin protein family in human cartilage is connexin 43 (Cx43, expressed by the *GJA1* gene) (17). Apart from the role of gap junctions in mesenchymal cell differentiation, connexin hemichannels also seem to be involved in chondrocyte mechanotransduction (18). However, the role of gap junctional communication during the different stages of chondroprogenitor differentiation and its overall contribution to cartilage tissue formation is still less well defined. This is important in considering the appropriate culture platform, *i.e.*, hydrogel-based or microtissues, for the different cell types.

This study was, therefore, aimed at comparing the differentiation potential of human articular chondrocytes and BM - MSCs in hydrogel and micromass culture systems with a particular focus on influence of gap junction-mediated cell-cell interactions on *in vitro* chondrogenesis. We employed sodium alginate hydrogels and micromass pellets as 3D differentiation models that either restricted or supported direct intercellular contacts. We also used a pharmaceutical inhibitor of gap junction activity to test the importance of this form of cell-cell communication during various stages of chondroprogenitor differentiation and the formation of cartilaginous tissue *in vitro*.

Materials and Methods

Cell isolation and expansion

Articular cartilage was collected with institutional ethics approval from consenting patients (4 male donors, age 26-50 years) either undergoing limb amputations or reconstructions of the anterior cruciate ligament. Cartilage specimens were frozen in Optimal Cutting Temperature compound (OCT, Sakura, Finetek, Tokyo, Japan) for histological analysis. Chondrocytes were isolated from full-thickness cartilage of femoral condyles or grooves as described previously (19). Chondrocytes were propagated on tissue culture plastic (3,000

cells/cm^2) in chondrocyte basal medium (low-D-glucose Dulbecco's Modified Eagle Medium with 4 mM L-alanyl-L-glutamine, 1 mM sodium pyruvate, 10 mM N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES), 0.1 mM non-essential amino acids, 50 U/mL penicillin / 50 µg/mL streptomycin (Pen/Strep) (all from Life Technologies, Carlsbad, CA), 0.1 mM L-ascorbic acid 2-phosphate and 0.4 mM L-proline (both Sigma Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT). Cells were used in subsequent experiments after passage two (~5-6 population doublings).
Mesenchymal stromal cells were isolated from bone marrow aspirates obtained under appropriate ethical approval from the iliac crest of consenting patients (1 female + 2 male donors, age 22-30 years) undergoing spinal fusion surgery. Total bone marrow cells were plated in α-MEM supplemented with 10% FBS, Pen/Strep and 1 ng/mL Fibroblast Growth Factor 2 (FGF-2) (Millipore, Billerica, MA). BM - MSCs were initially seeded at 100 cells/cm^2, subsequently plated at 1,000 cells/cm^2, and used between passages 3-4.
All cells were maintained at 37˚C in a humidified 5% CO_2/95% air CO_2 incubator with medium refreshed twice per week. Cells were passaged when subconfluent with 0.25% trypsin with 1 mM ethylenediamine tetraacetic acid (trypsin/EDTA) (Life Technologies) at 37˚C for 5 min and counted by trypan blue (Life Technologies) exclusion in a haemocytometer.

Chondrogenic differentiation

Chondrogenic potential was evaluated using either micromass pellet or alginate hydrogel cultures. Pellets of both chondrocytes and BM - MSCs were formed by centrifugation (19). Briefly, cells resuspended in a chondrogenic media were transferred into V-bottom shaped, 96-well microplates (AXYGEN Scientific, Union City, CA, USA) at 2 x 10^5 cells in 0.2 mL media per well and centrifuged at 600x*g* for 5 min. After 3 days, cells had condensed into micropellets that could be lifted from the bottom of the wells and transferred into 48-well plates containing 0.5 mL chondrogenic media. Cells were also encapsulated in 2% w/v sodium alginate (Pronova UP LVG, Novamatrix/FMC BioPolymers, Sandvika, Norway) at 10^7 cells/mL (20). Briefly, the cell alginate suspension was transferred into wells (height: 1.5 mm, diameter: 4 mm, volume: ~20 µL) of a stainless steel custom-made mold, placed between sterile filter papers and soaked in 102 mM $CaCl_2$ for 10 min. Hydrogel constructs were then washed with serum-free basal medium twice for 5 min and transferred into 48-well plates containing 0.5 mL chondrogenic media. Cells were cultivated in serum-free high-glucose chondrocyte basal medium supplemented with 1.25 mg/mL bovine serum albumin, 10^{-7} M dexamethasone, (all from Sigma), 1% v/v ITS, and 10 ng/mL transforming growth factor type beta 1 (TGF-β1) (both Life Technologies) and kept under reduced (5%) oxygen tension in a ProOx C-Chamber (Biospherix, Redfield, NY) inside a cell culture incubator. In order to reversibly block gap junction activity, 80 µM of 18-α glycyrrhetinic acid (18αGCA, Sigma) was added to the chondrogenic media of selected constructs at various time intervals. This concentration for 18αGCA is well below of what has been reported to be toxic to human fibroblasts in long-term experiments but potent enough to reduce intercellular junctional communication in albumin-containing cultures (21). Cultures were maintained for up to 9 days with media refreshed every three days and conditioned media stored at -80°C for further analysis. Photographs of pellets were taken with a phase contrast microscope (NIKON, Tokyo, Japan) at day 4 and 9 of culture and pellet sizes measured using ImageJ ((National Institutes of Health, USA).

Quantification of glycosaminoglycans (GAG) and DNA

Cells in constructs were harvested for GAG and DNA quantification by incubation with 0.5 mg/mL proteinase K (Life Technologies) in a phosphate buffer (20 mM Na_2HPO_4, 30 mM $NaH_2PO_4.H_2O$, 5 mM EDTA, pH 7.1) at 60°C overnight.
The GAG content of conditioned media as well as digested alginate samples and cell pellets was quantified using the 1,9-dimethylmethylene blue dye (DMMB) assay (pH 1.5) (22). Absorbances at 525 and 595 nm were measured in a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA), and the concentrations were calculated using the ratio of absorbances, compared to a quadratic standard curve prepared from chondroitin sulfate C (Sigma). Data from the first media change were excluded to allow for diffusion of unbound alginate from gels groups.
The Quant-iT™ PicoGreen® dsDNA assay (Life Technologies) was used to quantify DNA content in digested samples, with fluorescence (485 nm excitation, 520 nm emission) measured using a POLARstar OPTIMA fluorescence micro-plate reader (BMG Labtech, Offenburg, Germany).

Ribonucleic acid (RNA) extraction and real-time reverse transcriptase-polymerase chain reaction (qRT-PCR)

To measure absolute expression levels of selected genes of interest, qRT-PCR was used. RNA was isolated using the PureLink™ RNA Micro Kit (Life Technologies) according to manufacturer's instructions which included mechanic disruption of cell pellets with pestles and dissolution of alginate hydrogels in 10 mM

HEPES, 50 mM sodium EDTA and 150 mM NaCl for 10 min, followed by sample homogenization with a 20 gauge needle and the removal of genomic DNA by on column treatment with DNase I (Life Technologies). First strand complementary DNA (cDNA) synthesis was performed using the SuperScript™ III first-strand synthesis supermix for qRT-PCR (Life Technologies) with no more than 300 ng of total RNA in 20 µL reactions following the manufacturer's protocol.
Primers were either used as published previously for *COL1A1*, *COL2A1*, *COL10A1*, *ACAN, RPL13A and B2M* (23) or designed using PrimerBank (24) and Primer BLAST (NCBI, Bethesda, MD) for *GJA1* (5'→3' F: GACCGGATAGTCAAGTTCGTAGC, R: GCAGGAGCTGTCCACGTAG), and *TBP* (5'→3' F: GAGCCAAGAGTGAAGAACAGTC, R: CATCACAGCTCCCCACCATATT). All PCR reactions were done in duplicates in 10 µL volumes in a 7500 PCR system (Applied Biosystems, Foster City, CA). Reactions contained 1 X SYBR® Green PCR Master Mix (Applied Biosystems), 200 nM of each forward and reverse primers and 0.3 µL of undiluted cDNA and started with 10 min denaturation at 95ºC followed by 40 cycles of 95ºC for 15 s and 60ºC for 30 s. The cycle threshold (Ct) value of each gene was normalized to the geometric mean of the housekeeping genes *B2M*, *RPL13A* and *TBP* using the comparative Ct method ($2^{-\Delta Ct}$).

Immunofluorescence staining analysis

To determine the expression of proteins of interest, samples from alginate constructs, pellets and native cartilage were frozen in OCT (Sakura) and sectioned. Fresh-frozen sections were fixed in 100% cold acetone for 15 min and then hydrated in 50 mM $BaCl_2$ / 100 mM Tris HCl (pH 7.3) buffer for 1 h. Antigen retrieval was only included in the collagen I and II staining procedures by applying 0.1% w/v hyaluronidase (Sigma) in phosphate buffered saline (PBS) for 20 min at 37°C. Sections were then probed overnight at 4°C in PBS with 2% donkey or goat serum (2% serum/PBS; Jackson ImmunoResearch, West Grove, PA) and one of the following antibodies: anti-collagen I (mouse IgG_{2a}, 1:300, I-8H5, MP Biomedicals, Solon, OH), anti-collagen II (mouse IgG_1, 1:200, II-II6B3, Developmental Studies Hybridoma Bank (DSHB)), anti-aggrecan (mouse IgG_1, 1:300, 969D4D11, Life Technologies), anti-connexin 43 (rabbit IgG, 1:200, C6219, Sigma) or isotype controls (rabbit IgG, ABCAM/Sapphire, Redfern, NSW; mouse IgG, Jackson; both 1:1000). Subsequently, samples were incubated in 2% serum/PBS with either a mix of AlexaFluor®488-labelled goat anti-mouse (IgG_1-specific) and AlexaFluor®594-labelled goat anti-mouse (IgG_{2a}-specific) or AlexaFluor®488-labelled donkey anti-mouse IgG and AlexaFluor®594-labelled donkey anti-rabbit IgG (all minimal x-reaction with other species, 1:150, Jackson) and 5 µg/mL 4′,6-diamidino-2-phenylindole (DAPI, Sigma) for 1h at room temperature. Images were captured with fixed exposure times on an Axio Imager.A1 microscope with epi-fluorescence attachment (Carl Zeiss, Jena, Germany).

Adenosine-5'-triphosphate (ATP) assay

To quantify ATP released into media, samples were taken at day 9 of chondrogenic culture, heated for 5 min at 95°C to inactivate ATPases and stored at -80°C. The ATP Determination kit (Life Technologies) was used according to manufacturer's instructions and luminescence was measured in 10 cycles of 2 s detections using the POLARstar micro-plate reader, immediately after addition of the luciferase reaction mix to the media samples. ATP concentrations were calculated from averaged luminescence measurements compared to a quadratic standard curve of ATP provided with the assay kit and further normalized to the average DNA content of samples from each treatment group and donor at day 9 of culture.

Statistical analysis

Statistical analyses were performed using SPSS Statistics v18 (IBM, Somers, NY). To test for differences between the cell culture types as well as control and 18αGCA-treated samples in alginate or pellet models, data from gene expression, biochemical and ATP assays were transformed using the binary logarithm to improve normality distribution of the data sets and tested by analysis of variance (ANOVA) using a general linear model including Dunnett's T3 post *hoc test* for multiple comparisons between groups. To test the effect of intermittent blocking of gap junctional communication on chondrogenic differentiation, gene expression data from day 3, 6 and 9 of pellet cultures were subjected to ANOVA using a general linear model including Tukey's HSD *post hoc* test. Statistically significant differences were considered to be present at $p<0.05$.

Results

Effect of cell-cell interactions and gap junction blocking on gene expression during chondrogenic differentiation

To study the importance of cell-cell interactions during differentiation of chondroprogenitor cells *in vitro*, expanded chondrocytes and BM - MSCs were cultured in hydrogel or micromass pellets, which either restricted or allowed for extensive direct cell-cell interactions, respectively. Additionally, gap junctional communication was blocked using the pharmaceutical compound 18αGCA. After 9 days of differentiation culture, the expression of the chondrogenic marker collagen type II (*COL2A1*) was significantly lower in 18αGCA-treated chondrocytes in both culture types compared to untreated controls (p=0.03, Fig. 1a). However, blocking gap junctions reduced the mRNA levels of aggrecan (*ACAN*), another chondrogenic marker, in chondrocyte pellets (p=0.02) but not in alginate cultures (p=0.19, Fig. 1b). Similar to *COL2A1*, transcription of the dedifferentiation marker collagen type I, *COL1A1*, was hampered by 18αGCA in chondrocyte alginate (p=0.002) and pellet cultures (p=0.01) in comparison to controls (Fig. 1c). Chondrocytes exhibited decreased transcription of collagen type X (*COL10A1*), a marker for tissue hypertrophy and calcification, only when cultured in pellets in the presence of a gap junction blocker (p=0.02, Fig. 1d). The gene expression of the major gap junction protein (*GJA1*) connexin 43 was elevated in untreated chondrocyte pellets compared to hydrogel cultures (p=0.01), but decreased only in pellets upon 18αGCA treatment (p=0.004) (Fig. 1e). In MSCs, gap junction blocking reduced mRNA levels of *COL2A1* (p=0.009) and *ACAN* (p=0.01) in alginate cultures compared to their respective controls, and although similar trends were observed in pellets, they failed to reach significance (*COL2A1*: p=0.3; *ACAN*: p=0.39) (Fig. 1f,g). Transcription levels of both *COL1A1* and *COL10A1* were not significantly different between 18αGCA-treated MSC alginate and pellet samples and control groups (Fig. 1h,i). In line with the chondrogenic markers, *GJA1* expression was only lower in treated MSC alginate cultures ($p<0.0001$) versus controls but unchanged in pellets in response to gap junction blocking (p=0.52, Fig. 1j). The mRNA levels of the matrix molecules (*COL2A1*, *ACAN*, *COL1A1*, *COL10A1*) were not significantly different between untreated alginate and pellet cultures in either chondrocytes or MSCs (Fig. 1).

Effect of cell-cell interactions and gap junction blocking on DNA and matrix production during chondrogenic differentiation

To study the importance of cell-cell interactions and gap junctional communication on matrix production of chondroprogenitor cells *in vitro*, we compared the DNA and GAG production of chondrocytes and BM - MSCs cultured for 9 days in hydrogel or micromass pellets in the presence or absence of 18αGCA. Blocking of gap junctions decreased DNA content (p=0.005), GAG per DNA (p=0.02) and the GAG in media (p=0.03) as well as the size ($p<0.001$) of chondrocyte pellets compared to untreated groups (Fig. 2a-d). Similarly, GAG per DNA in constructs as well as the GAG secreted into the media was also lower in chondrocyte alginate cultures treated with 18αGCA versus controls (Fig. 2b-c). Yet, DNA content was not different between these two groups. The GAG per DNA accumulated by chondrocytes over 9 days was higher in untreated alginate cultures in comparison to pellets (p=0.03; Fig. 2b). In contrast to chondrocytes, DNA amounts, GAG per DNA, GAG in media and pellet sizes were not different between BM - MSCs cultured in the presence or absence of the gap junction blocker in either alginate or pellet cultures (Fig. 2e-h). DNA and GAG synthesis of BM - MSCs was also comparable between alginate and pellet control cultures (Fig. 2e-g).

Effect of cell-cell interactions and gap junction blocking on protein expression

The deposition of extracellular matrix after 9 days in differentiation culture under varying levels of gap junctional communication was assessed by probing with antibodies specific for collagen type I and II. Exposure to the gap junction blocker reduced the staining intensity for both the chondrogenic collagen type II and the dedifferentiation marker collagen type I in all culture models with chondrocytes (Fig. 3a-d). Compared to control pellets, sections from 18αGCA-treated chondrocyte pellets revealed overall higher cell density and less matrix at day 9 of culture and unusual islands of intense collagen I staining (Fig. 3c,d). Similar to chondrocytes, 18αGCA-treated MSCs showed less immunoreactivity against collagen I compared to untreated controls (Fig. 3e-h). Collagen type II staining was weak in both culture types with BM - MSCs and only limited to single cells in the untreated controls (Fig. 3e-h). Inherent differences in cell seeding densities between alginate and pellets cultures preclude a direct comparison of their matrix qualities based on immunofluorescence staining.

Sections from the various groups at day 3 and 9 of culture were also probed with antibodies for the cartilaginous matrix protein, aggrecan, and the gap junction protein connexin 43 (Fig. 4). All groups revealed immunoreactive regions for aggrecan that were confined to the extracellular matrix. At day 9, untreated alginate and pellet controls with chondrocytes and BM - MSCs showed slightly more intense

immunoreactivity for aggrecan and connexin 43 compared to 18αGCA-treated groups (Fig. 4b,c,e,f,h,i,k,l). There were no clear differences in connexin 43 staining between day 3 and 9 of chondrocytes or BM - MSCs in alginate cultures (Fig. 4a,b,g,h). However, chondrocyte pellets showed more intense, punctate staining throughout all regions at the earlier compared to the later time point, which coincided with higher cell density and lower immunoreactivity for aggrecan at day 3 compared to day 9 (Fig. 4d,e). In contrast, with BM - MSCs, areas of more intense, clustered staining for connexin 43 appeared to be localized to the outer rim of pellet sections at both time points (Fig. 4j,k). Control sections from the superficial and middle/deep zone of human native cartilage revealed extracellular matrix positive for aggrecan as well as localized cellular staining for connexin 43, that was seemingly less intense compared to pellet cultures (Fig. 4m,n). Stainings with IgG isotype control antibodies from mouse and rabbit proved negligible unspecific background reactivity (Fig. 4o).

Effect of cell culture models and gap junction blocking on extracellular ATP levels

Connexin hemichannels have been implicated in the release of ATP into the extracellular environment (18). Therefore, we measured ATP levels in the conditioned media from chondrocytes and BM - MSCs cultured in alginate or pellets in the presence or absence of 18αGCA between days 6 to 9 and normalized these values to the average DNA content of samples from each treatment group and donor at day 9 of culture. Gap junction blocking decreased the amount of ATP in media normalized to DNA of chondrocyte alginate ($p=0.007$) and pellet cultures ($p=0.002$; Fig. 5a). The same effect was observed for BM - MSCs in alginate cultures ($p=0.014$) but not in pellets ($p=0.89$; Fig. 5b). ATP amounts in media per DNA were not different between alginate and pellet control groups of chondrocytes ($p=0.18$) nor BM - MSCs ($p=0.7$; Fig. 5a,b).

Discussion

Intercellular communication via gap junctions is an important driver of mesenchymal condensation in early chondrogenic differentiation (12). Yet, it is far from clear if direct cell-to-cell contacts and the exchange of messenger molecules through gap junctions also have an essential role in the differentiation of chondroprogenitor cells in *ex vivo* engineered cartilage constructs. Furthermore, scaffold architecture and composition not only play a critical role in modulating chondrogenic differentiation and diffusion of nutrients (25, 26), but also in controlling cell distribution in 3D (27). Yet the influence of 3D cell distribution and concomitant cell-cell communication on the differentiation capacity of chondroprogenitor cells offered by either high density microtissue versus more discrete hydrogel culture platforms for cartilage tissue engineering has also not been well established.

Our data show that differentiating chondrocytes or BM - MSCs in micromass cell pellets brought no advantage over alginate hydrogel cultures in terms of the cell's chondrogenic marker expression and GAG synthesis. Our findings are in agreement with other studies, in which neither human chondrocytes (28) nor human BM - MSCs (29) showed clear differences in chondrogenic marker expression when differentiated in either pellet or alginate cultures. This suggests that promoting direct intercellular contacts may not be critical for the differentiation of chondroprogenitor cells *in vitro*.

Despite this, we still found that blocking gap junctional intercellular communication using the pharmaceutical inhibitor 18-α glycyrrhetinic acid (18αGCA) negatively affected chondrogenic differentiation, as evident in a reduction in GAG synthesis and in the expression of the important chondrocyte marker, collagen type II (Fig. 1+2). Interestingly, 18αGCA treatment did not altogether abolish the expression of chondrogenic marker molecules and seemed to affect chondrocytes and BM - MSCs to a different extent depending on the cell culture model. Aggrecan mRNA levels of chondrocytes, for example, were only significantly reduced in pellets but not in alginate, which may be explained by the higher connexin 43 expression in the former compared to the latter (Fig. 1). In contrast, while there was a strong impact on *COL2A1* and aggrecan expressions in BM - MSC alginate cultures, reduction in these markers was less severe in BM - MSC pellets. It should be noted that there were large donor-to-donor variations for BM - MSCs in terms of their the chondrogenic potential, which may explain the high standard deviations in the mRNA and biochemical data sets and could be - at least in part - to blame for the difficulties to detect significant effects for this cell type. Another interesting difference between the two cell types was that 18αGCA treatment had the most profound effect in the first few days of chondrocyte differentiation in pellets, yet, chondrogenesis of BM - MSCs appeared to be disturbed only past day 3 (Supplementary Fig 1). This has also been observed in mesenchymal cells from chicken limb buds, which only showed a discernable response to 18αGCA treatment 48 h after initiation of chondrogenic differentiation (30). It could, therefore, be speculated that BM - MSCs, the more immature chondroprogenitor cell type as evidenced by relatively low *COL2A1* and *ACAN* expression at the start of the differentiation, respond only to impediments of their gap junction function after the chondrogenic phenotype has been established. It is interesting to note in this regard that connexin 43 protein expression was, indeed, highest at day 3 of chondrocyte pellet culture whereas BM - MSC pellets appeared to exhibit higher connexin 43 levels at day 9 than day 3 (Fig. 4). In addition, our results and those

of other groups indicate that connexin 43 mRNA and protein levels decrease in cells in response to 18αGCA at concentrations higher than 20 μM (31). This raises the question about the mechanism of action of the gap junction blocker 18αGCA, which does not seem to be fully elucidated to date. We only observed minor effects on cell numbers in treated cultures. DNA levels in 18αGCA samples were not different to controls except for chondrocyte alginate cultures, in which case DNA quantities of the 18αGCA group were still at similar levels as measured at day 0 of culture (Fig. 2). Some evidence points to the incorporation of 18αGCA into the cell membrane, where it disrupts the formation of gap junction plaques and possibly induces a conformational change of connexin closing the gap junctions (32). Furthermore, it has been proposed that glycyrrhetinic acid interferes with the interaction between connexin 43 and phosphatases or kinases altering connexin phosphorylation and leading to reversible closure of gap junction and reduced gap junction intercellular communication (31, 33). Therefore, the main mode of actions of 18αGCA is most likely a combination of decrease in connexin expressions and interference with opening of gap junctions.
A role for gap junctional intercellular communication in cell differentiation has been established for a number of cell types, including epithelial and mesenchymal cells (30, 34). Our study shows for the first time that chondrogenesis is even hampered by 18αGCA in alginate cultures with already limited cell-cell contacts. This suggests that the effect of gap junction-mediated crosstalk on chondrocyte differentiation is mainly exerted through the formation and activity of hemichannels. Hemichannels are aqueous membrane pores that allow for the controlled release of small messenger molecules (*e.g.*, ATP, prostaglandin E_2, ions) for autocrine/paracrine cellular communication in response to a range of stimuli (35). In chondrocytes, connexin hemichannels seem to be involved in the release of intracellular ATP into the extracellular space after cyclic loading, thereby, modulating purinergic and calcium signaling pathways (18, 36). In our study, we found, indeed, reduced ATP levels in the media of almost all groups treated with a gap junction blocker compared to their controls (Fig. 5). Only media samples from BM - MSC pellets showed no differences for this nucleotide, which may be explained by ATP release from dying or dead cells that were observed at relatively high levels in these cultures (Fig. 2e). In summary, our data clearly point to a broader, important role for hemichannel activity and ATP release in chondrogenic differentiation even in the absence of mechanical stimuli. Indeed, a study on the mouse chondrogenic cell line ATDC5 revealed synchronized ATP oscillations during early differentiation that appeared gap junction-dependent and positively affected pre-chondrogenic condensation (37). However, the signals driving gap junctional ATP release in the absence of cyclic mechanical cues still remain elusive.
In this regard, it is tempting to speculate about the potential use of small molecule messengers, such as ATP, as relatively inexpensive media supplements to improve quality of engineered cartilage constructs. However, it may not be as simple as continuously adding ATP to culture media. When human BM - MSCs and bovine chondrocytes were differentiated in agarose hydrogels, the presence of ATP did not enhance matrix contents and brought only minor improvements to mechanical properties of the constructs (38). Therefore, the role for extracellular ATP in chondrogenic differentiation is likely to be complex and requires further investigation.

Conclusions

Alginate hydrogel and microtissue culture systems are equally suited to study the *in vitro* differentiation of articular chondrocytes and bMSCs. Immediate cell-to-cell interactions offered by micromass culture do not play a significant role in the *in vitro* chondrogenesis of expanded human chondrocytes and human BM - MSCs compared with hydrogel culture. Therefore, restricting direct intercellular communication through scaffold architecture should not affect the chondrogenic potential of isolated cells in scaffold–based cartilage repair strategies. However, our study demonstrates to our knowledge for the first time the importance of connexin hemichannels in gap junction-mediated crosstalk between chondrocytes during differentiation that goes beyond their involvement in chondrocyte mechanotransduction, and appears to control the release of small molecules, such as ATP, for autocrine and paracrine signaling. Further studies need to address the specific role for connexin hemichannels in coordinating or regulating chondrogenic differentiation and identify the specific cues that drive hemichannel activity. This will give new insights in the complex processes of chondrogenesis and potentially reveal new important factors and strategies to improve current approaches in cartilage tissue engineering.

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Author Disclosure Statement

No competing financial interests exist.

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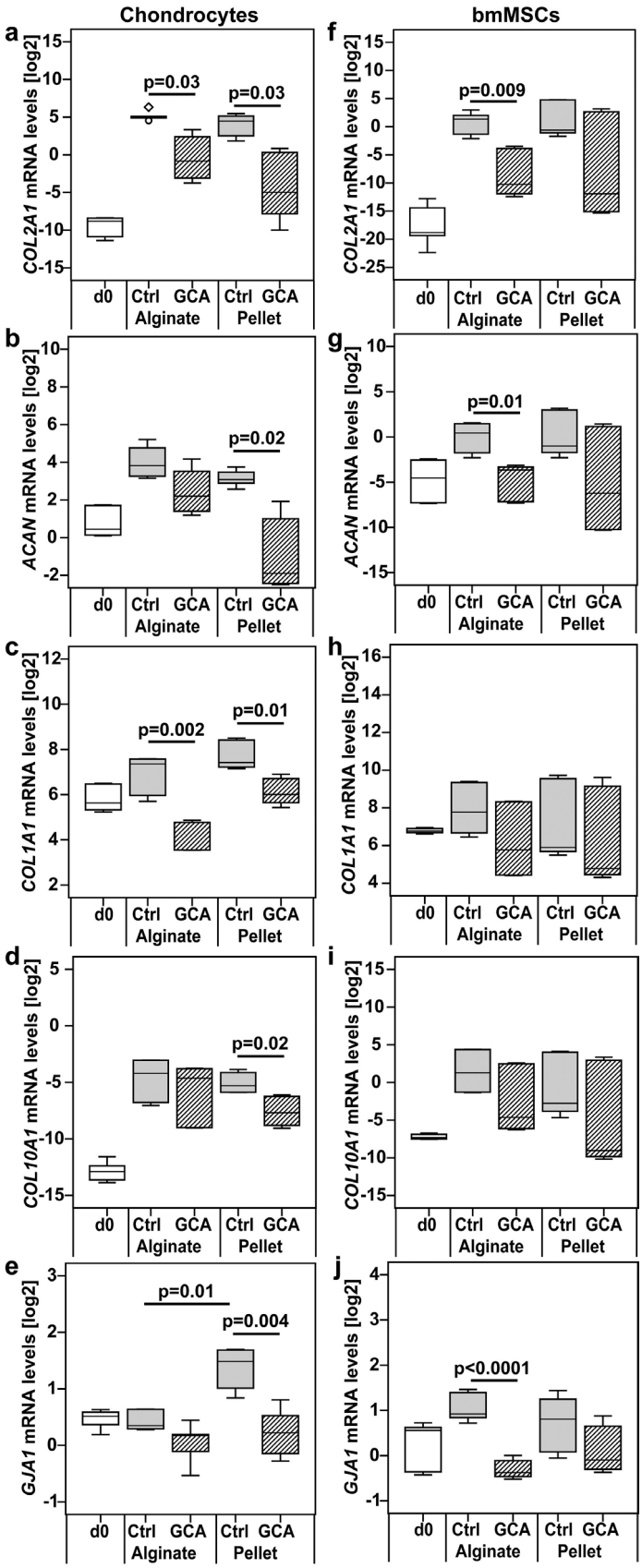


Fig 1: Gene expression levels of (a, f) collagen II (*COL2A1*), (b, g) aggrecan (*ACAN*), (c, h) collagen I (*COL1A1*), (d, i) collagen X (*COL10A1*) and (e, j) connexin 43 (*GJA1*) in (a-e) human articular chondrocytes or (f-j) BM - MSCs following treatment with the gap junction blocker 18αGCA. Cells were differentiated in alginate hydrogels or pellet cultures in the absence (Ctrl) or presence of 18αGCA (GCA). The gene expression levels were measured at day 0 (d0) and day 9 of culture with qRT-PCR, then normalized to housekeeping genes using the comparative deltaCt method and transformed into the binary logarithm (log2). For clarity, statistical significance only shown for comparisons between 18αGCA-treated groups and controls as well as alginate Ctrl versus pellet Ctrl (n=6 from three donors of each cell type).

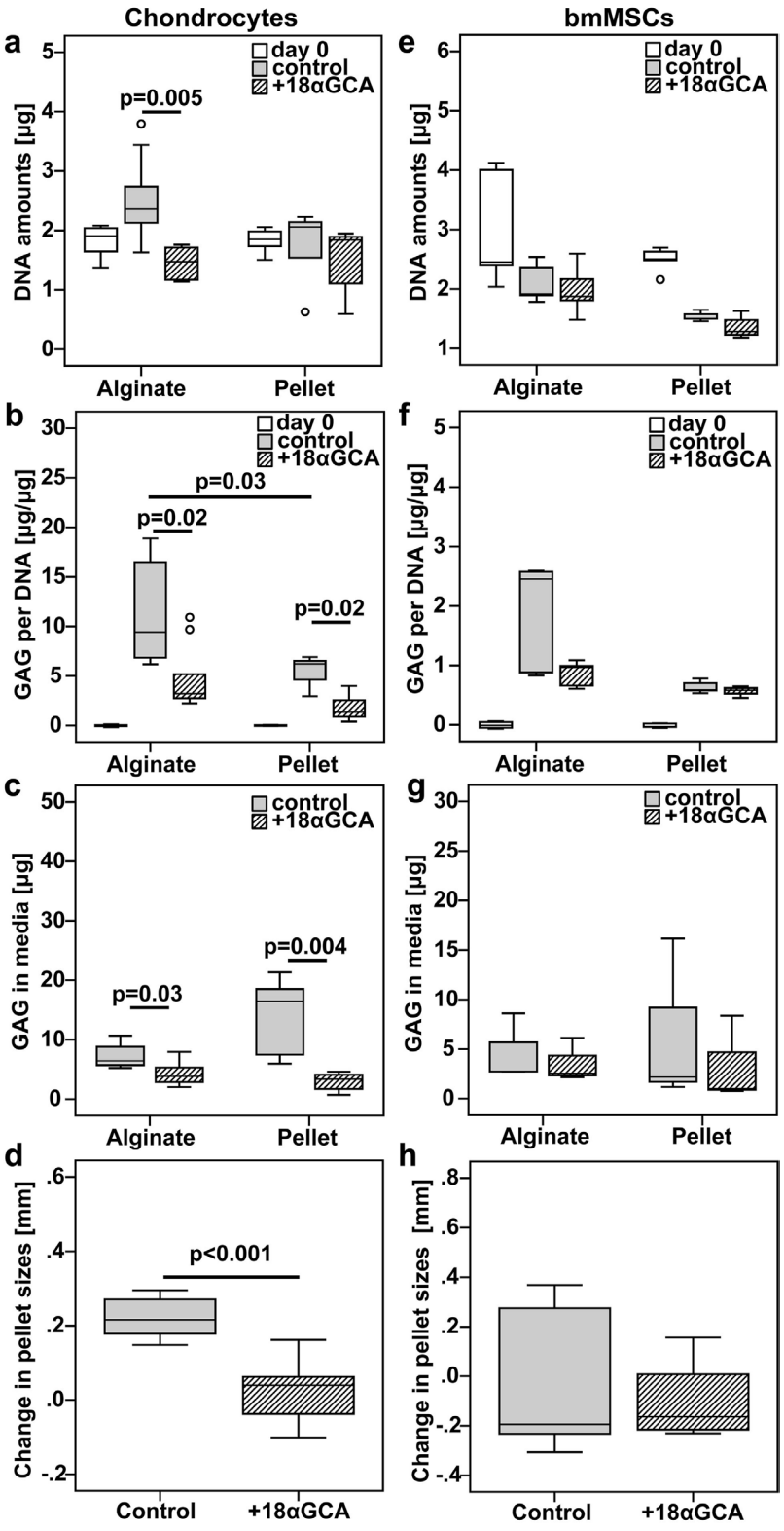


Fig. 2: Effect of gap junction blocking on DNA and GAG synthesis of (a-d) human articular chondrocytes and (e-h) BM - MSCs in alginate hydrogel and micromass pellet cultures. Cells of both types were cultured under identical chondrogenic conditions in the absence (control) or the presence of the gap junction blocker 18-α glycyrrhetinic acid (+18αGCA). The amounts of (a, e) DNA and (b, f) GAG per DNA were measured in constructs collected before (day 0) and at the end of the culture period. The cumulative amount of (c, g) secreted GAG was measured in conditioned media from day 6 and 9 of culture. The (d, h) change in pellet sizes was measured based on photographs taken with a phase contrast microscope at day 4 and 9 of culture. For clarity, statistical significance only shown for comparisons between 18αGCA-treated groups and controls or alginate Ctrl versus pellet Ctrl (chondrocytes from 3 donors: n=7 pellets, n=9 alginate constructs, n=15 pellets for size measurements; MSC from 2 donors: n=5 pellets or alginate constructs, n=9 pellets from 3 donors for size measurements).

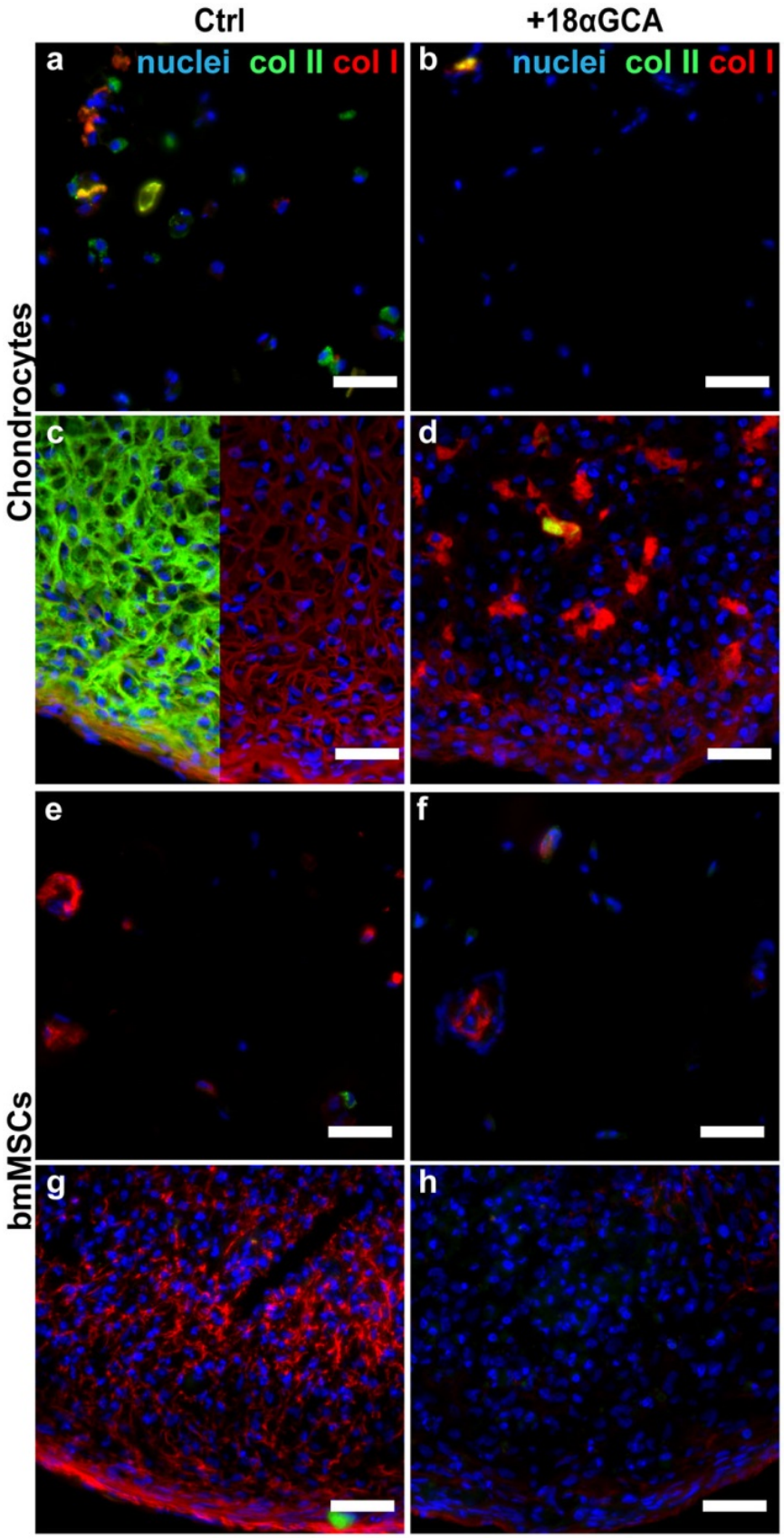


Fig. 3: Expression of collagen type II and type I from (a-d) human articular chondrocytes and (e-h) BM - MSCs in (a, b, e, f) alginate hydrogel and (c, d, g, h) micromass pellet cultures. Cells of both types were cultured under identical chondrogenic conditions in the (a, c, e, g) absence (Ctrl) or the (b, d, f, h) presence of the gap junction blocker 18-α glycyrrhetinic acid (+18αGCA). Representative samples were processed for fresh-frozen tissue immunohistology at day 9 and simultaneously probed with antibodies against collagen type II (col II, green) or collagen type I (col I, red) and counterstained with DAPI (blue). In panel c, col II signal was omitted in the right half of the image for better visibility of col I staining. Scale bars = 50 µm.

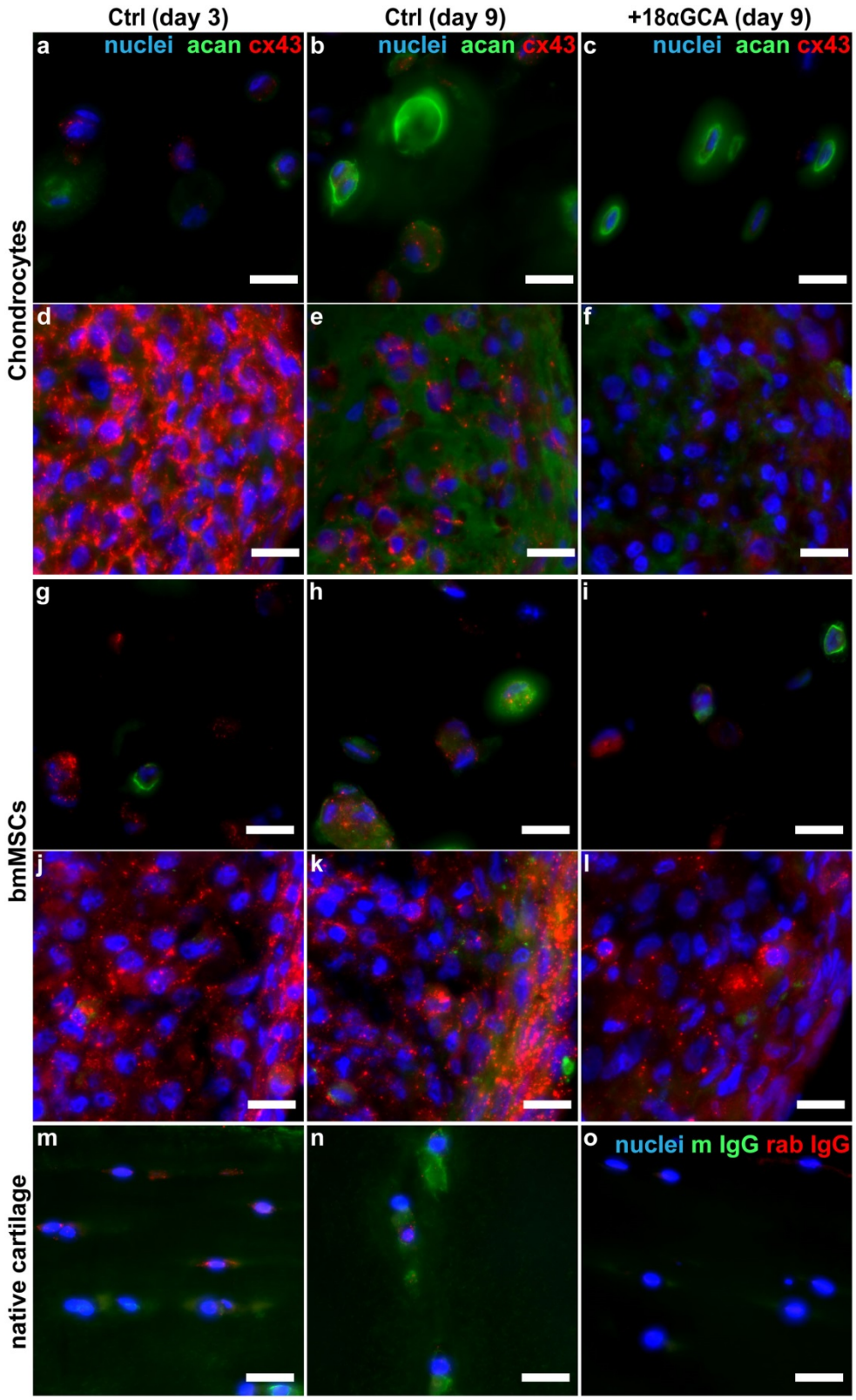


Fig. 4: Expression of aggrecan and connexin 43 from (a-f) human articular chondrocytes and (g-l) BM - MSCs in (a, b, c, g, h, i) alginate hydrogel and (d, e, f, j, k, l) micromass pellet cultures. Cells of both types were cultured for (a, d, g, j) 3 days or (b, c, e, f, h, i, k, l) 9 days under identical chondrogenic conditions in the (a, b, d, e, g, h, j, k) absence (Ctrl) or the (c, f, i, l) presence of the gap junction blocker 18-α glycyrrhetinic acid (+18αGCA). Representative samples were processed for fresh-frozen tissue immunohistology and simultaneously probed with antibodies against aggrecan (acan, green) or connexin 43 (cx43, red) and counterstained with DAPI (blue). Sections from the (m, o) superficial or (n) the middle zone of native human articular cartilage either (m, n) stained for aggrecan/connexin 43 or (o) probed with unspecific antibodies against mouse IgG (m IgG) and rabbit IgG (rab IgG) are shown for comparison. Scale bars = 20 µm.

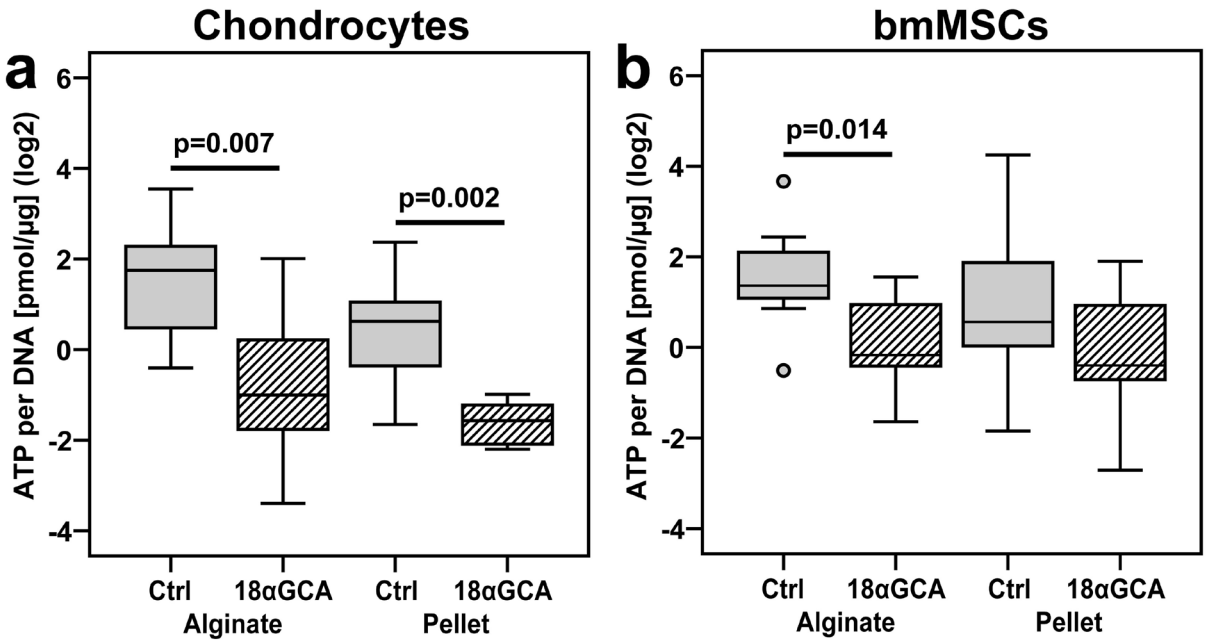
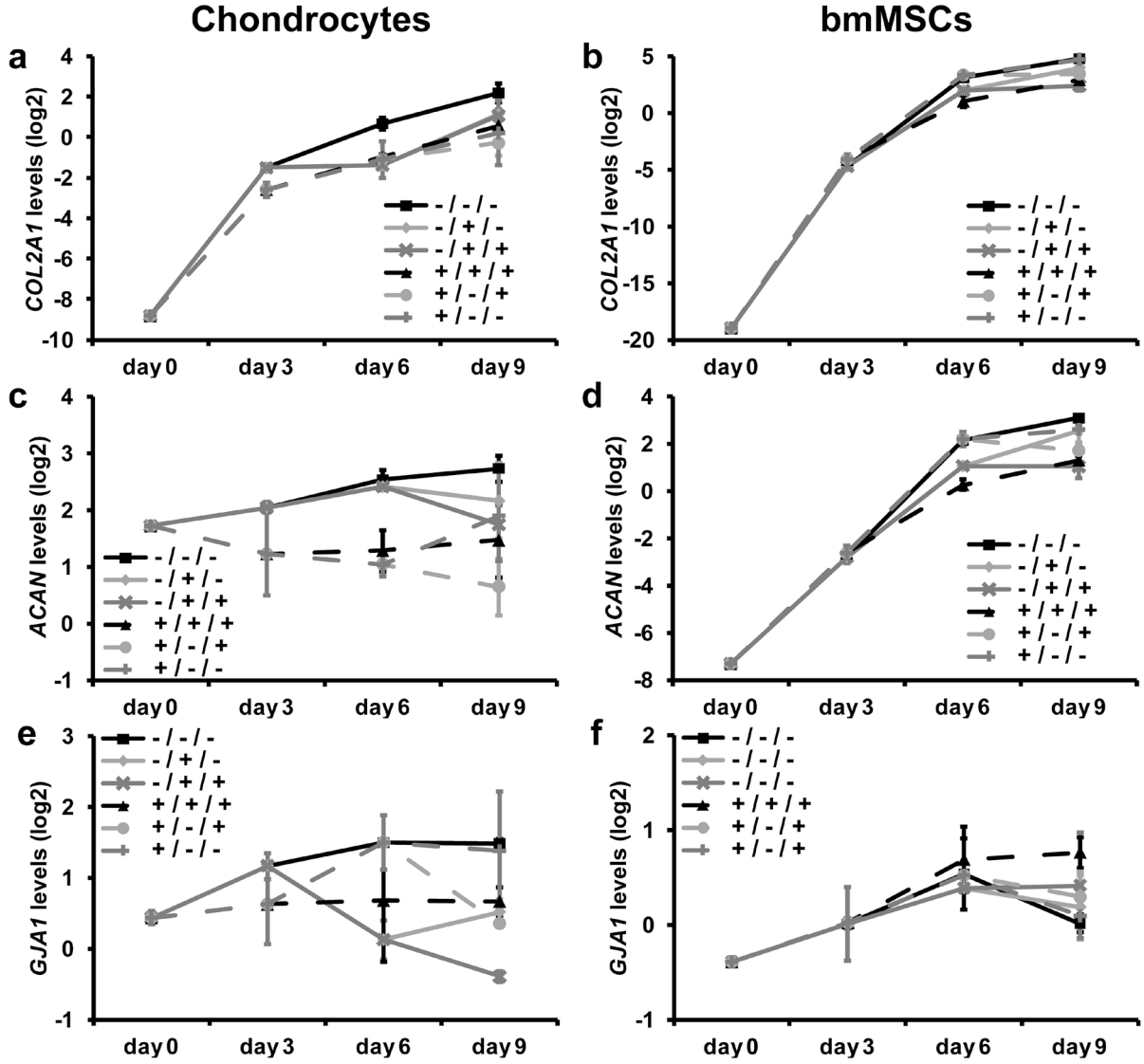


Fig. 5: Effect of gap junction blocking on ATP secretion of (a) human articular chondrocytes and (b) BM - MSCs in alginate hydrogel and micromass pellet cultures. Cells of both types were cultured under identical chondrogenic conditions in the absence (Ctrl) or the presence of the gap junction blocker 18-α glycyrrhetinic acid (+18αGCA). The amount of secreted ATP was measured in conditioned media from day 9 of culture, normalized to the average DNA content of samples from each treatment group and donor at day 9 of culture and transformed into the binary logarithm (log2). (n=8-12 from two donors of each cell type).



Supplementary Fig 6: Gene expression levels of (a, d) collagen II (*COL2A1*), (b, e) aggrecan (*ACAN*) and (c, f) connexin 43 (*GJA1*) in (a-c) human articular chondrocytes or (d-f) BM - MSCs following intermittent treatment with the gap junction blocker 18αGCA. Cells were differentiated in pellet cultures over nine days and media was changed every three days supplemented either with (+) or without (-) 18αGCA. The different groups received 18αGCA treatments in various intervals as indicated in the graphs. Control pellets were cultured in the complete absence (- / - / -) or in the presence (+ / + / +) of 18αGCA for the entire period. (mean ± standard error, n=2 per time point and condition, one donor).

Effect of intermittent blocking of gap junctional communication on chondrogenic differentiation

To determine if the importance of gap junction communication changes with time in pellet differentiation cultures, chondrocyte and BM - MSC pellets were alternately exposed to the gap junction blocker 18αGCA at various three day intervals during a nine day culture period. Chondrocyte pellets receiving 18αGCA over the entire nine days of culture (+/+/+) had lower transcript levels for *COL2A1* (p=0.01) and *ACAN* (p=0.01) than untreated pellets (-/-/-; Supplementary Fig 1a,b). This was also observed with BM - MSCs (Supplementary Fig 1d,e; *COL2A1*: p=0.003; *ACAN*: p<0.001). However, these two treatment groups were not significantly different in respect to *GJA1* expression (Supplementary Fig 1c,f; chondrocytes: 0.09; MSC: 0.35). There appeared to be cell-specific differences in when gap junction blocking had the greatest effect on pellet differentiation. Chondrocyte pellets with gap junction communication blocked in the first 3 days tended to show reduced expression of these markers at day 6 even when 18αGCA was removed from the culture media between day 3 and 6 (e.g.: -/- versus +/- at day 6; *ACAN*: p=0.01; Supplementary Fig 1b). In contrast, MSC pellets that only received gap junction blockers in the first three days of culture but not thereafter recovered quickly and increased expression of chondrogenic markers compared to pellets treated with 18αGCA between days 3 and 6 (e.g.: +/- versus -/+ at day 6; *COL2A1*: p=0.03; *ACAN*: p=0.02; Supplementary Fig 1d,e). In general, chondrogenic differentiation of BM - MSCs in pellets was more affected

by disruptions in gap junctional communication between day 3 and 9 as shown by reduced *ACAN* (p=0.005) and *COL2A1* (p=0.01) mRNA levels compared to untreated controls at day 9 of culture (-/-/- versus -/+/+; Supplementary Fig 1d,e). There was no such effect on chondrocyte pellets, and mRNA levels were comparable at day 9 irrespective of when the intermittent gap junction blocking occurred (Supplementary Fig 1a,b).